THE DEPARTMENT OF CELL AND MOLECULAR BIOLOGY
Karolinska Institutet, Stockholm, Sweden

BIOPHYSICAL REGULATION OF CELL FUNCTION: THE YIN AND YANG OF THE MICROENVIRONMENT

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Karolinska Institutet

Stockholm 2014

On the cover: Human induced pluripotent stem cell-derived neuroepithelial-like stem cells stained for actin (green) and DNA (blue). Image by Dr Richard Mills.

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Published by Karolinska Institutet. Printed by Åtta.45 Tryckeri AB.

ISBN 978-91-7549-495-1

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Biophysical Regulation of Cell Function: The Yin and Yang of the Microenvironment

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Hillarpsalen, Institutionen för Neurovetenskap, Retzius väg 8, Karolinska Institutet

Fredagen den 14 mars, 2014, kl 09.30

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Hold fast to dreams, for if dreams die,
life is a broken-winged bird that cannot fly.

Langston Hughes

ABSTRACT

From embryonic development to tissue regeneration and disease progression, the human body is continuously subject to mechanical stresses. Physical forces are increasingly recognized as major microenvironmental cues that control tensional homeostasis in tissues. Cells constantly receive and translate physical cues into biological messages, which in turn dictate cell shape, state and function. While much is known about biochemical signaling, many of the mechanisms that drive cell outcome in response to biophysical influences remain to be uncovered.

Here we have investigated biophysical regulation of cell function. The goal was to gain a deeper understanding of fundamental principles that govern cell behavior in response to physical stimuli. To carefully recapitulate signaling in the *in vivo* microenvironment, we utilized a battery of tools that stem from the field of bioengineering. We used conjugated polymers to develop a novel neural stem cell culture substrate with anchored growth factors to promote cell self-renewal. Upon an electrochemical switch, growth factor presentation was reversed, which initiated cellular differentiation along the neuronal lineages. This electroactive material allowed for temporal control of growth factor presentation, increased growth factor stability and a closer reflection of biological signaling during brain development *in vivo*.

In addition to temporal changes in growth factor presentation, mechanical stiffness of tissues is also dynamically altered over time. Cells sense and respond to the mechanics of their substrate - be it the extracellular matrix, neighboring cells or artificial matrix in cell culture. Using biologically relevant elastic substrates to study cell function *in vitro* has proven beneficial, as the *in vivo* microenvironment usually is much softer than rigid plastic dishes. Stiffened tumor stroma is a hallmark of cancer and understanding mechanosensitive pathways involved in the onset of cancer is key in identifying strategies for cancer treatment. We have therefore investigated the role of matrix stiffness in Notch signaling in breast cancer cells. This signaling pathway is a highly conserved cell-to-cell communication system that regulates cell fate in development and disease. Aberrant Notch signaling in breast cancer has been found to correlate with invasion and patient outcome. Our results show that we can tune cell stiffness and migration by regulating Notch activity and matrix stiffness. We propose an opportunity to target the cancer cell/microenvironment interface instead of the Notch pathway itself in the development of cancer therapies.

Finally, we have studied the role of nanoarchitecture of ephrin ligands in Eph receptor activation. Eph/ephrin signaling is a cell-to-cell communication pathway, which regulates cell migration and proliferation. Dysregulation of this pathway has been associated with a multitude of human diseases, including breast cancer. Here, we developed a new tool based on DNA origami, which allows for precise positioning of ephrin ligands on DNA at the nanoscale. We found that Eph receptor activation and downstream events are regulated by ephrin spatial distribution. This work contributes to our understanding of how physical cues in the form of ligand presentation impact breast cancer cell behavior. Ultimately, elucidating the mechanisms involved in biophysical regulation of cell function is necessary to understand cellular dysfunction and diseases.

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I. **Lundin V**, Herland A, Berggren M, Jager EWH, Teixeira AI (2011) Control of Neural Stem Cell Survival by Electroactive Polymer Substrates. PLoS One 6(4):e18624.

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* Equal contribution

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
AFM	Atomic force microscopy
bHLH	Basic helix-loop-helix
BLBP	Brain lipid binding protein
CNS	Central nervous system
CSL	CBF1/Suppressor of Hairless/Lag-1
DLL	Delta-like ligand
ECM	Extracellular matrix
EGF	Epidermal growth factor
Eph	Erythropoietin-producing hepatocellular carcinoma
ESC	Embryonic stem cell
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
iPSC	Induced pluripotent stem cell
JAG	Jagged
MAP	Mitogen-activated protein
MLC	Myosin light chain
MMP	Matrix metalloprotease
MSC	Mesenchymal stem cell
NC	Nano-caliper
NICD	Notch intracellular domain
Nrarp	Notch-regulated ankyrin repeat protein
NSC	Neural stem cell
PEDOT	Poly(3,4-ethylenedioxythiophene)
PLA	Proximity ligation assay
PPy	Polypyrrole
PSS	Poly(styrenesulfonate)
ROCK	Rho-associated protein kinase
RTK	Receptor tyrosine kinase
SH2	Src-homology2
SHH	Sonic hedgehog
T-ALL	T-cell acute lymphoblastic leukemia

INTRODUCTION

THE CELLULAR MICROENVIRONMENT

Cellular functions are carefully orchestrated by signals present in the microenvironment. These signals include growth factors and cytokines, the extracellular matrix, oxygen and calcium levels as well as cell-cell interactions. Together, these signals act in a tightly controlled manner over space and time to provide stimuli that regulate key functions, such as cell survival, proliferation and differentiation.

Direct signaling between cells and the extracellular matrix or between neighboring cells in tissues is a key mechanism by which cells receive cues that direct their state and fate. These interactions are largely mediated by specialized receptor proteins present in the cell membrane. Activation of receptors is dependent on the presence and binding of ligands, which initiates a sequence of molecular switches that transduce a signal from the exterior to the interior of the cell. Ligands are often soluble molecules secreted by cells that can act both locally and over long distances. Tethered ligands, however, require direct physical contact, either between cells and the extracellular matrix or between juxtaposed cells. Ligands bind specifically to the extracellular domain of the receptor, usually triggering a conformational change in the intracellular domain of the receptor, which attracts signaling proteins or activates enzymatic activity in the receptor itself. The transduced signal is often amplified, producing multiple intracellular messages. Ultimately, a message conveyed to the inside of the cell mediates a physiological response that regulates cell function.

Throughout the lifetime of a higher-order organism, cells receive and translate external mechanical cues into biological messages, which dictate cell form, fate or function. While biochemical signals in the cellular microenvironment have been extensively investigated, studies regarding the effects of mechanical signals were for a long time neglected. Nevertheless, the human body is continuously subject to forces, from innate forces, such as gravity, to those induced by exercise and movement, resulting in stretching and relaxing of tissue. As such, mechanical stresses are constantly present on a cellular level and it is now widely accepted that they are major conductors in the regulation of cell function. For instance, when microenvironmental forces are applied to cells, cells have to meet force with resistance. This force balance is a fundamental mechanism in maintaining tensional homeostasis, part of the microenvironmental yin and yang of cells and tissues.

In my thesis work, I have investigated signaling pathways that rely on direct physical contact, in the context of biophysical regulation. Below I will describe these pathways, and later, their relevance in development and disease. The common strategy in each of the systems studied was to recapitulate signals present in the *in vivo* microenvironment to better understand their influence on cell function. Each study was designed to answer fundamental biological questions using cell engineering approaches and resorting to a broad array of materials and technologies.

FGF SIGNALING

Fibroblast growth factors (FGF) are polypeptide ligands that execute their actions by binding to tyrosine kinase receptors. These receptors regulate key cellular functions ranging from germ cell maturation and development to homeostasis and tissue repair. Dysregulation of the FGF signaling pathway has been well documented in many diseases, including developmental disorders and cancer.[1] The 18 mammalian secreted FGFs share a common core region of 120-140 amino acids, which are divided into subfamilies based on their sequence similarities in their amine (N-) and carboxy (C-) terminals.[2] The four different fibroblast growth factor receptors (FGFR1-FGFR4) are single-pass transmembrane receptors with a split tyrosine kinase domain in the intracellular part of the receptor and three immunoglobulin-like domains in the extracellular region. A fifth receptor, FGFR5, lacks the intracellular tyrosine kinase domain and is less understood.[3] Moreover, tissue specific alternative splicing events, both of the ligands and receptors, generate increased ligand-receptor specificity.

FGF ligands have a heparan sulfate glycosaminoglycan binding site and are readily sequestered to heparan sulfates in the extracellular matrix (ECM). Heparan sulfates are long chains of repeating disaccharide units, chemically similar to heparin, and are components of the ECM, where they augment in FGF-FGFR binding. Specifically, the negatively charged heparin sulfates form a complex with canonical FGF, which then facilitate FGFR dimerization by binding to the positively charged lysines and arginines present in the heparin binding sites on the ligands.[4] Variation in the sequence of receptor, ligand and heparan sulfate leads to a diverse range of binding between these molecules, which ultimately gives rise to high biological diversity.[5,6] Importantly, heparan sulfate glycosaminoglycans act as a storage reservoir for FGF and stabilize the ligand against degradation.[7] Dimerization of the FGF/FGFR/heparan sulfate complex leads to a conformational change of the intracellular domain of the receptors, which initiates transphosphorylation of the tyrosine kinase domain and C-terminal. This process then activates subsequent downstream signaling pathways, such as the Ras, Akt or the protein kinase C pathways.[1] The work presented here focused on FGF2, also known as basic FGF. FGF2 is involved in synaptic formation, inflammation and neuron-glia interaction.[8] It is also involved in defining rostro-caudal identity in the neural tube during development.[9] Importantly, FGF2 is a neurogenic factor that regulates neural stem cell proliferation and differentiation through its interaction with FGFR1, both during development and in the adult.

NOTCH SIGNALING

The Notch signaling pathway is an evolutionary conserved signaling system present in all metazoans, mediating cell fate choices by direct cell-cell contact through proteins on cell membranes. It is clear from the literature that this pathway is significant in the development, homeostasis and pathology of all three germ layers and their derivatives. Ligand-receptor binding triggers Notch receptor proteolysis, and unlike most other signaling pathways, the released intracellular receptor domain translocates into the nucleus, where it is physically involved in gene expression activation. Despite being a molecularly relatively simple signaling system, the Notch pathway elicits very diverse responses that vary over time and space in a cell context-dependent manner. Due to the repeated use of the Notch signaling pathway throughout the lifespan of an organism, disruption thereof has shown diverse and severe effects. As such, developing mice with defective Notch signaling show general growth retardation and embryonic lethality before E10.5.[10] Moreover, Notch dysregulation is associated with multiple diseases, including cancer, where it can act either as an oncogene or as a tumor suppressor depending on the cellular context, again reflecting the versatility of the Notch signaling system.[11]

The Notch signaling pathway includes a series of catalytic events of the receptor that ultimately leads to transcriptional regulation. The Notch receptor first undergoes furin cleavage at the S1 site in the *trans*-Golgi network by a convertase, resulting in a heterodimeric protein that is recruited to the plasma membrane. The single-pass Notch receptor (Notch 1-4 in mammals) consists of an intracellular PEST domain on the C-terminal, a transcription activation domain (TAD), two nuclear localization signals (NLS) flanking the six ankyrin (ANK) repeats, a RBP-J Associated Molecule (RAM) domain and a transmembrane domain that connects the intracellular domain to the extracellular domain. The extracellular region consists of a heterodimerization domain (HD), three cysteine rich LIN-12-Notch repeats (LNR) and 29-36 EGF-like repeats, repeat 11 and 12 being necessary for ligand binding.[12] LNR and HD make up the negative regulatory region (NRR), which is unmasked upon ligand binding.

Notch binds any of the five canonical ligands, Jagged (JAG1 and JAG2) or Delta-like (DLL1, DLL3, DLL4) on juxtaposed cells. Ligand binding is followed by two catalytic steps: A Disintegrin and Metalloprotease (ADAM) cleaves the receptor extracellularly (S2 cleavage) and gamma-secretase cleaves the receptor within the transmembrane domain (S3 cleavage), liberating the Notch intracellular domain (NICD).[13,14] The NICD then translocates into the cell nucleus where it binds the DNA-binding protein CSL (CBF1, Suppressor of Hairless, Lag-1; also known as RBP-Jκ). In the absence of Notch signaling, CSL functions as a transcriptional repressor, together with corepressors such as CtBP, HDAC and Hairless. However, upon NICD binding to CSL, repressors are released and coactivators, including Mastermind-like1 and histone acetyltransferases HAT/p300, are recruited to the transcription complex, which initiates transcription of Notch target genes,[15] as shown in Figure 1. Immediate target genes of active Notch signaling are the basic helix-loop-helix proteins (bHLH) Hes and Hey, which in turn function as transcriptional repressors. Other target genes, for example of relevance to cancer, are the cell cycle associated genes Myc, cyclin-D1 and p21, as well as genes linked to epithelial-mesenchymal transition, such as Slug.[16] Moreover, Notch

activates its own expression and that of its ligands, as well as transcription of its own negative regulators Notch-regulated ankyrin repeat protein (Nrarp) and Deltex-1, providing an intrinsic negative feedback loop.[17,18,19]

While endocytosis generally functions as a means to down-regulate receptor signaling, the Notch signaling system instead requires endocytosis for pathway activation and downregulation of the receptor. However, endocytosis is not only necessary in the receptor-expressing cell, but notably, the ligand must also be endocytosed into the signaling cell for Notch to be activated in the receiving cell.[20] For years, the mechanistic connection between ligand endocytosis and receptor activation remained unclear, until it was demonstrated that ligand-receptor binding creates a physical pulling force that reveals the NRR and allows ADAM to cleave at the S2 site, a prerequisite for S3 cleavage and release of the NICD.[21] The requirement for a biomechanical force in Notch signaling activation has sparked a hypothesis that Notch is in fact mechanosensitive. Therefore, Notch signaling in mechanosensing has received increasing attention, for instance in the fields of angiogenesis and blood formation.

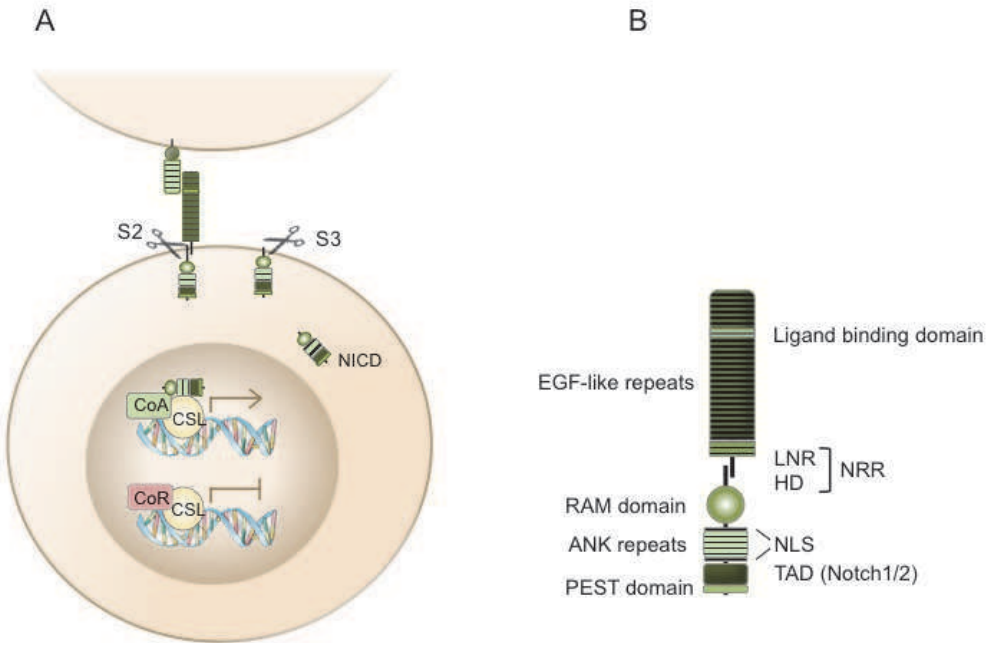

Figure 1. Notch signaling. (A) Upon ligand binding, the Notch receptor is cleaved by ADAM at the S2 site, followed by cleavage by S3 γ-secretase in the cell membrane. The liberated active NICD translocates to the nucleus, where corepressors (CoR) are released and NICD binds to the DNA-binding protein CLS with coactivators (CoA), which initiates transcritption. (B) The Notch receptor consists of an intracellular PEST, TAD, two NLSs, an ANK domain, a RAM domain and a transmembrane domain. The extracellular part of the receptor consists of a HD, three LNR and 29-36 EGF-like repeats, where 11 and 12 are necessary for ligand binding. LNR and HD make up the NRR, which protects S2 from cleavage in the absence of ligand.

EPH/EPHRIN SIGNALING

Receptor tyrosine kinases (RTKs) are one type of ligand-dependent cell membrane receptors with intrinsic enzymatic activity, which is involved in development and disease. Erythropoietin-producing hepatocellular carcinoma (Eph) receptors are the largest class of RTKs and bind specifically to their corresponding ephrin ligands. The Eph/ephrin cell-to-cell communication pathway is involved in many embryonic developmental processes, including axon guidance, cell migration and the formation of tissue boundaries. Moreover, Eph/ephrin signaling is also involved in adult tissue homeostasis, for instance by regulating proliferation and migration of progenitor cells in the intestine, one of few adult tissues with a highly proliferative stem cell pool.[22] Dysregulation of this pathway has been implicated in many forms of cancer. Interestingly, Eph/ephrin signaling has shown both tumor suppressing and tumor promoting characteristics, depending on the tumor type and stage. These observations are in line with the complexity of this signaling pathway in normal tissue.

Eph receptors are divided into two classes: EphA and EphB. There are nine different mammalian EphAs and five different EphBs. EphAs bind one of the six mammalian ephrinA ligands, whereas EphBs preferentially bind one of the three ephrinBs, although some cross-class interaction is also known to occur. Eph receptors are single-pass transmembrane proteins consisting of an intracellular PDZ domain-binding site, a sterile alpha motif (SAM) domain and a tyrosine kinase domain, which is linked to the plasma membrane through a juxtamembrane region. In the extracellular domain of the receptor there are two fibronectin-type III repeats, a cystein rich domain and the ligand-binding domain. The ephrins consist mainly of the Eph binding domain in their extracellular region. While ephrinB has an intracellular region with a PDZ domain, the ephrinA ligands are glycophosphatidylinositol-linked proteins and lack an intracellular domain.[23]

Binding of ephrins to the receptors induces transphosphorylation by the kinase domains of the tyrosine residues on the intracellular domains of Eph. Two conserved phosphorylation sites on tyrosine residues in the juxtamembrane domain of the receptor are of particular importance, since they release the auto-inhibitory interaction between the juxtamembrane and the kinase domain. This allows the receptor to take on a more stable and active conformation, thus enabling further phosphorylation of tyrosine residues on the kinase domain, as illustrated in Figure 2. Some of these phosphorylated tyrosines recruit signaling molecules containing src-homology2 (SH2) domains.[24,25] Other signaling molecules that are recruited to the receptor complex are guanine nucleotide exchange factors for Rho family GTPases (GEFs) and PDZ domain-containing proteins. The different signaling proteins recruited upon kinase activity will mediate various downstream signaling cascades and cell functions, for example cell migration through RhoA or RhoG via the GEF Ephexin.[26]

In contrast to other common ligand-receptor signaling systems, Eph/ephrin interaction activates both forward and reverse signaling, meaning that signaling cascades can be triggered both in the receptor-expressing cell (forward signaling) as well as in the ligand-expressing cell (reverse signaling). Reverse signaling involves phosphorylation of the ephrinB cytoplasmic tail by Src family kinases, and recruitment

of signaling effectors. However, reverse signaling through EphrinA, which lacks a cytoplasmic domain, requires the association of transmembrane signaling partners, such as p75 or Trk/B, to trigger phosphorylation of Src family kinases and phosphoinositide 3-kinases.[27] Eph/ephrin bi-directional signaling is a well-studied phenomenon and is a key mechanism behind many known functions, such as axon migration and growth cone collapse, as well as spine and synapse formation.[28,29,30]

To build upon this complexity, receptor clustering is key in transmitting a downstream signal. Ephrin ligands usually form clusters in the cell membrane of the signaling cell, which cross-links Eph receptors on neighboring cells. This is in contrast to several other RTKs, in which the ligand has to bind simultaneously to only two receptors. Thus, soluble recombinant monomeric ephrin ligands are inefficient in activating the Eph receptor. Instead, to activate forward Eph signaling and study downstream responses *in vitro,* ephrin-Fc fusion proteins are clustered with an anti-Fc antibody, which creates higher-order ephrin clusters. Whereas recombinant extracellular ephrin-Fc fusion proteins that interfere with endogenous Eph/ephrin binding can be used to block reverse signaling, extracellular Eph-Fc fusion proteins can be used to stimulate reverse signaling, and simultaneously block Eph forward signaling. The ability to induce receptor activation in one direction, while inhibiting the other, is of great interest in the development of cancer therapeutics that target the Ehp/ephrin signaling pathway.

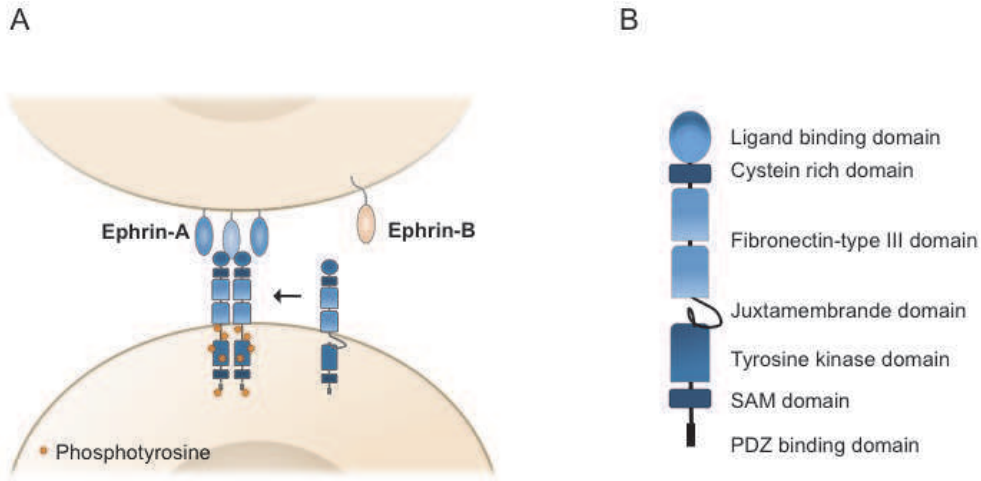

Figure 2. Eph/ephrin signaling. (A) Eph receptors binding to ephrin ligands on opposing cells initiates receptor clustering and autophosphorylation of tyrosine residues in the intracellular receptor domain. (B) The intracellular domain of the Eph receptor consists of a PDZ binding domain, a SAM domain, the tyrosine kinase domain and a juxtamembrane domain. The extracellular domain of the receptor has two fibronectin-type III repeats, a cystein rich domain and the ligand-binding domain.

MECHANOTRANSDUCTION

Mechanical stresses are ever present in the microenvironment and influence form and growth of every cell and tissue, from gastrulation to organ regeneration. Forces are key in the most fundamental functions of our bodies, such as heartbeat, the circulatory system and in muscles, tendons and bones. On a smaller scale, mechanical forces exist as main components in the cellular microenvironment and drive basic cellular functions, including proliferation, differentiation, adhesion, migration and apoptosis. Cells constantly feel and respond to their immediate surrounding, whether it is a cell culture dish, nearby cells or matrix. In fact, the ECM was long viewed as a scaffolding structure which main function was to maintain tissue morphology, and that the cells in this scaffold received information from their environment in the form of chemical cues, like growth factors and cytokines. Today it is well understood that cells are poised to respond to mechanical stimuli arising in the microenvironment, such as the ECM, which in fact is a highly dynamic and versatile structure. Moreover, cells themselves generate internal forces by contractility that regulate essential cell functions, such as chromosome rearrangement during mitosis, organelle transport and migration. Whereas much is known about the chemical signals that influence cell state, less is known about the physical cues and how these are translated, or transduced, into biological information. Mechanotransduction refers to the process used by cells to respond to their three-dimensional (3D) environment. Specifically, it is the mechanism by which cells convert mechanical cues in their surrounding to biochemical signals, which in turn steer cell function through gene expression or other cell behavior. The role of forces in tissue remodeling and development was studied already in 1892 and gave rise to Wolff's law, which states that the form and function of bones are in direct correlation to the stress imposed upon them. However, it is only with recent development of new technologies that this field has generated such great attention and insight into the biomechanics of cells and tissue.

Cells have highly dynamic and complex machinery that regulates intracellular forces. The cytoskeleton, consisting of a coordinated network of microtubules, intermediate filaments and actin filaments, constitutes a crucial part of this machinery. Actin makes up the actin cortex, a thin filament layer just beneath the plasma membrane that provides mechanical support. Together with non-muscle myosin II, actin generates cell contractility forming the actomyosin network that connects various regions of the cell membrane, as well as the membrane to the nucleus.[31] Furthermore, the intracellular actin cytoskeleton is physically linked to transmembrane integrins that bind specific peptide sequences, like RGD, in the ECM on the outside of the cells. Upon application of force, integrins cluster with adaptor proteins to form focal adhesions, which are large protein multicomplexes that provide points of adhesion between cells and the ECM. Importantly, forces that arise in the ECM create stress on the focal adhesions when the actin cytoskeleton applies an opposing force from the inside of the cell. The same applies in the reverse direction: forces generated inside the cell create stress on the focal adhesions resulting in an opposite, reactive force in the ECM. Cells in a soft microenvironment with a pliable ECM fail to develop mature focal adhesions.[32] Similarly, transmission of an applied force is inhibited when contractility through myosin is blocked.[33]

Focal adhesions do not merely provide cell-ECM adhesion, nor does the actomyosin network function only to regulate external-internal force balancing. They also have the important function of conveying messages to the cells that ultimately influence cell form and function. Focal adhesions are thus important biochemical signaling centers, containing a large number of adaptor proteins that via stress fibers transduce information necessary for proper cell function. Some of these proteins include talin, vinculin and paxillin, which physically link the integrins to the actomyosin network. Further, maturation of focal adhesions attracts a number of signaling molecules, including focal adhesion kinase (FAK), p130Cas, Src, small GTPase Rho, ERK and Akt, which trigger phosphorylation and force-induced structural rearrangements that initiate signaling transduction and contractility.[34,35] Typically, phosphorylation of myosin light chain (MLC) by MLC kinase promotes an actin-myosin interaction that is balanced by MLC phosphatase. These events are regulated by the Rho-effector Rho-associated protein kinase (ROCK), which controls MLC phosphorylation levels.

A study in which researchers applied nano-Newton forces to individual focal adhesions in fibroblasts showed that the application of a minute force resulted in relaxation and recovery of contractility in the cell over a longer time period than that of the force applied.[36] Another study showed that cells pulled harder on an adhesion that resisted the cells' pulling forces.[37] These results demonstrate how focal adhesions regulate a highly dynamic feedback system involving external forces, cytoskeletal rearrangements, reinforcement at the site of adhesion and transduction of the signal. Importantly, cells constantly produce, remodel and degrade components of the ECM, creating a reciprocal relationship between the ECM and cells. The resulting changes in the ECM will in turn influence behavior of nearby cells. Additionally to cell-ECM interaction and regulation, mechanotransduction between cells work in a similar fashion, as cells naturally also pull on their neighbors. Actin-mediated cell-cell contacts are formed through the cadherin protein family that make up adherence junctions. Similarly to focal adhesions, interaction of cadherins induces myosin II activation, which promotes actin stabilization and recruitment of additional cadherins and signaling proteins, triggering signal transduction.[38] Moreover, stress activates mechanically gated ion channels, which decrease or increase ion fluxes upon applied force.

From initial biophysical input, through adhesion points, to the cytoskeleton and to the final cellular output, there are multiple mechanisms and opportunities for versatility in the long chain of events involved in signaling transduction. The heterodimeric integrins are formed from an α and a β subunit and each integrin combination has its own binding specificity. Integrins bind several ECM proteins and have specific signaling properties. Physical properties of the ECM, including spatial arrangement, porosity, topography and stiffness, regulate focal adhesion architecture and subsequent recruitment of signaling molecules. Further, actomyosin filaments as well as focal adhesions are dynamic complexes with constant protein association and dissociation, leading to continuous competition for binding sites between various signaling molecules. Force-induced structural rearrangements; posttranslational modifications of proteins, such as phosphorylation and de-phosphorylation; and the activity and subcellular localization of different key players, such as Rho GTPase, focal adhesion kinase, Src family members, mitogen-activated protein (MAP), protein kinase C kinases

or integrin-linked kinase, further introduce versatility in the system and the ultimate biological outcome. As for every other signaling system in the human body, each of the events in the chain of mechanotransduction is under extreme tight regulation and is dependent on cell type, location and timing. The rapid recognition of mechosignaling has inspired research to identify alternative signaling pathways that are involved in mechanotransduction. In this work, we investigate the possibilities of Notch and Eph/ephrin signaling being components of mechanosensitive pathways.

CELLS CAN FEEL TOO

The ECM is a dynamic structure that undergoes constant stiffness changes through production, matrix crosslinking and proteolysis during development and disease progression. The fact that cells fail to develop mature focal adhesions on soft substrates, along with decreased levels of RhoA and FAK, suggest that myosin activity and cellular contractility is directly altered by substrate stiffness.[39] Furthermore, forced RhoA activity in cells on soft substrates promotes a cell phenotype resembling that of a cell growing on a stiff substrate. Although not entirely understood, cells are believed to feel the elasticity of their surrounding using similar mechanotransduction mechanisms as when a force is applied to cells. Interestingly, endogenous cell contractility can be modulated without applying external forces by simply changing the mechanical stiffness of the substrate against which the cells pull. Scientists have long been making use of both natural and synthetic materials to elicit phenotypes that are otherwise hard to capture *in vitro*. Mimicking the natural elasticity of cells and tissue when studying mechanotransduction is key since these physical cues are completely lost on rigid surfaces. In fact, cell culture dishes are at least a million times stiffer than the softest tissues of our body. Generally, cells grown on soft substrates look round with poorly arranged stress fibers, whereas cells grown on stiff material polarize and spread, form focal adhesions and well-aligned stress fibers.[40,41] Cells presented to substrates with both soft and stiff areas tend to migrate to the stiffer region, a process called durotaxis.[41,42]

Interestingly, several studies have shown that the proper substrate stiffness can directly tune cell state and fate, including maintenance of pluripotency, promotion of self-renewal, and direct lineage specification.[43–45] In particular, differentiation of bone marrow derived mesenchymal stem cells (MSCs) cultured on gels of varying stiffness was shown to depend on substrate elasticity. In this study, MSCs grown on stiff substrates promoted the expression of osteogenic markers, while intermediate substrate stiffness induced myogenic differentiation and the softest substrates promoted expression of neuronal markers.[45] Other work has demonstrated the promotion of neuronal maturation on soft substrates, compared to stiffer substrates.[46,47] The central strategy in these experiments was the same: to match the elasticity of the substrate to the *in vivo* tissue stiffness. These studies demonstrate that biophysical cues regulate cell shape, function and fate.

In addition to matrix elasticity, there are a multitude of biophysical factors in the microenvironment that affect cell function. Although outside the scope of this thesis, some examples that demonstrate the role of mechanotransduction in the regulation of cell function follow here. In addition to substrate stiffness, cell shape also drives

differentiation, as shown for human MSCs grown on micropatterns that allowed for control of cell spreading. Here, MSCs on small patterns became adipogenic, whereas MSCs on larger patterns became osteogenic. The cell shape-based lineage commitment was RhoA dependent, and direct manipulation of RhoA signaling was sufficient to drive MSC differentiation in either direction to become fat or bone cells.[48] Contact guidance, the phenomenon of directional growth of cells along substrate topography, has been widely studied on microfabricated patterns, showing that cells tend to elongate and align to ridges and grooves at the nanoscale with a dependence on groove width.[49,50] This does not only have implications in development but also in cancer, where aligned matrices are often found near tumors, providing support for migrating cells and promoting tumor cell invasion.[51] The effect of stress and strain on cell behavior is also well-characterized, both *in vitro* and *in vivo*, demonstrating the influence of stress and strain on development and homeostasis in tissues such as the lungs, kidneys, circulatory system and brain.

THE TUMOR MICROENVIRONMENT

WHEN CELLS LOSE TOUCH

While it is widely recognized that tumor cells receive soluble signals from nearby stromal cells, mechanical communication is also crucial for tumor cells. Consequently, the biophysical microenvironment plays a crucial role in cancer development and progression. When the mechanotransduction machinery is deregulated, for instance by mutations in the proteins involved in the downstream signaling pathway, force sensing will be altered and tissue homeostasis perturbed. Any other alterations within the cells or in the microenvironment, like the ECM, that change the transmission of forces can promote decreased or increased signaling transduction and lead to disease. Normal ECM function is tightly controlled by several regulatory mechanisms, which ensure proper composition and stiffness. A key mechanism is the production and activity of enzymes, such as matrix metalloproteases (MMPs), that remodel and break down components of the ECM. As such, dysregulation of these potent enzymes during aging or disease progression can have destructive consequences. Increased degradation of the ECM allows for greater motility of cells that subsequently break away and invade other tissue. For instance, matrix metalloprotease expression and activity is elevated in most types of cancer and correlates with invasion and metastasis. Moreover, abnormal composition, arrangement, topography and amount of ECM can cause, as well as be a cause of aberrant cell function. Hence, disorganization, reduced matrix turnover and increased matrix deposition are all hallmarks of cancer.

Tumors are most often much stiffer than normal tissue due to increased rigidity of the matrix. In breast cancer, the stroma has been found to display up to ten times higher stiffness compared to healthy tissue, which has been directly coupled with tumor cell invasion and progression.[52] *In vitro*, non-transformed mammary epithelial cells cultured in soft collagen 3D gels formed polarized, acinar structures, while those cultured in stiffer gels lost their polarization and showed an increase in proliferation and characteristics of a malignant phenotype.[53] Force-dependent aggregation and clustering

of integrins led to intensified stabilization of focal adhesions but disrupted adherence junctions in a Rho-ROCK-dependent manner. Reducing cellular tension by regulating Rho signaling reversed the malignant phenotype and inhibited tumor cell proliferation. Other studies have confirmed elevated integrin levels and signaling activity in breast cancer cells and similarly to Rho disruption, inhibition of integrin expression through genetic ablation resulted in decreased breast malignancy.[54]

SIGNALING IN BREAST CANCER

The Notch signaling pathway has been linked to many different human cancers. The Notch receptor was initially highlighted in humans as an oncogene in T-cell lymphoblastic leukemia (T-ALL) and it was later shown that Notch1 has mutations resulting in constitutive activity in over 50% of all T-ALL cases.[55] Further, ligand-independent Notch4 ICD expression has been discovered in murine mammary cancers, and Notch1 ICD has been shown to induce transformation of mammary epithelial cells *in vitro*.[56,57] Active forms of the Notch1 ICD have been found in several human breast cancer cell lines, where a decrease in Notch signaling levels reversed the transformed phenotype, and conversely, Notch activation in normal mammary epithelial cells induced transformation.[58] Numb, a key negative regulator of Notch signaling, is lost in around 50% of human breast cancers due to degradation. This correlated with tumor grade, and cell growth in these tumors could be suppressed by Notch inhibition.[59] Notably, elevated levels of Notch1 and the ligand Jagged1 in breast cancer tumors correlates with poor survival.[60]

Similarly to the Notch signaling pathway, Eph/ephrin signaling is also greatly implicated in cancer and expression levels of both ligand and receptor are correlated with cancer progression and patient outcome. For example, the receptor of interest in paper IV, EphA2, is overexpressed in many cancers, particularly in more than 40% of breast cancers.[61] In a large panel of breast cancer cell lines studied, there was an inverse relationship between ligand-receptor expression: expression of EphA2 was inversely correlated to the levels of ephrin-A1. Further, EphA2 suppression was induced upon ligand expression due to ligand-mediated receptor internalization and degradation. Transfection of EphA2 in normal epithelial cells was sufficient to induce transformation and further studies suggested that the observed oncogenic potential of EphA2 was exerted independently of ligand-induced receptor phosphorylation.[62]

Common for the Notch and Eph/ephrin signaling pathways is that they both can act as tumor promoters as well as tumor suppressors. Which of the two mechanisms that is used is cell-context dependent. Moreover, crosstalk with other signaling pathways is known to be involved in tumor promoting or suppressive functions. Notch has for instance been shown to crosstalk with other oncogenic signaling pathways, such as Wnt and Hedgehog, as well as with estrogen receptors. EphA2 interacts physically with the EGF receptor, which was shown to induce Erk and Rho GTPase activity in a ligand-independent manner, promoting tumor cell malignancy. On the other hand, EphA2 stimulation with ephrin-A1-Fc reduced Erk phosphorylation in tumor cell lines and inhibited transformation of mouse fibroblast cells.[63]

SIGNALING IN DEVELOPMENT

NEURAL DEVELOPMENT

Neurulation is the embryonic developmental process that leads to the formation of the neural tube, the precursor of the entire central nervous system (CNS). Neurulation involves a carefully orchestrated sequence of events that include induction and elongation of the neural plate along the length of the embryo, folding of the neural plate and neural tube closure in a zipper-like manner, during which it buds off from the overlying ectoderm. The rostral part of the neural tube develops into the forebrain, midbrain and hindbrain, whereas the rest of the neural tube forms the spinal cord.

When neurulation commences, a single layer of neuroepithelial cells with neural stem cell (NSC)-like properties start appearing in the ventricular zone at embryonic day (E) 8 in rodents. These cells are highly bipolar, with their apical side attached to the ventricle and a basal process stretching to the pial surface. During the expansion phase of the stem cell pool, the nuclei migrate in an apico-basal fashion called interkinetic nuclear migration, where they stay at the basal lamina during DNA replication and move to the apical-most area for cell division. During midgestation, the neuroepithelium thickens and the cells maintain their neuroepithelial properties, expressing for example the intermediate filament marker nestin. At the same time they become more elongated and start expressing astroglial markers, such as brain lipid binding protein (BLBP) and glial fibrillary acidic protein (GFAP). These cells are referred to as radial glia and undergo repeated asymmetric cell division, during which young neurons migrate basally, out of the ventricular zone, along the radial glia. A new germinal layer is formed, called the subventricular zone, harbouring proliferative basal progenitor cells. These cells maintain their proliferative capacity and make up the pool of neural stem cells in the adult. The cerebral cortex of the telencephalon is eventually formed from migrating neurons, which give rise to six cortical layers, with the youngest neurons found closest to the cortical surface. In the adult, proliferative activity of neural cells is limited to the dentate gyrus of the hippocampus and the lateral walls of the lateral ventricles.

Development of the CNS is an extremely intricate process, during which the cell state must be regulated under precise spatial and temporal control. Successful neural development is a result of exact coordination of cells' polar organization, cell cycle length, regulation by growth factor input, epigenetics, cell communication and so forth. These events work in concert to affect cells in a context-dependent manner. Together, these regulative mechanisms control cell fate switches, patterning, maintenance of the progenitor pool and brain size.

Patterning of the body axis occurs through the repeated use of specific molecular signaling mechanisms, which relay spatial and temporal information to progenitor cells. Just like the rest of the developing embryo, development of the CNS follows tight regulatory machinery, controlling the balance and timing of gene expression. For instance, morphogen gradients of signaling molecules specify positional identity to populations of progenitor domains. In the developing neural tube, cells are exposed to two opposing gradients of Wnts/bone morphogenic proteins (BMPs) and Sonic Hedgehog (SHH), the combination of which defines distinct progenitor domains in a

concentration-dependent manner. Moreover, cells in the developing CNS receive patterning information in an anteroposterior fashion, which regionalizes progenitors along the length of the body axis. In addition to Wnts, BMPs and SHH, FGFs are important in defining positional identity in the developing telencephalon.

At least ten different FGFs seem to have a role in brain development, from the earliest stages of neural induction to establishment of appropriate connectivity as well as in the adult brain. For example, at E9.5-10 of the developing mouse brain, the expression of FGF8 and FGF17 is highly regionalized in certain domains rostrocaudally in the brain. Further, whereas the two analogues FGF1 and FGF2 both are potent mitogens in the developing CNS, FGF2 is expressed in the cortex earlier than FGF1 and while FGF2 is expressed both in neuronal and non-neuronal cells, FGF1 expression is restricted to neuronal cell types.[64]

Although the focus of the study in this work was FGF signaling in NSCs, the approach used here has potential to be applied also with other signaling mechanisms, such as Notch or Eph. These pathways are also highly involved in the development of the CNS, as briefly described below.

The Notch signaling pathway plays a crucial role in making cell fate choices in CNS development. At the onset of neurogenesis, Notch inhibits differentiation of neurons by expressing the bHLH proteins Hes and Hey, which repress proneural genes, such as Neurogenin. However, once a cell acquires lineage commitment, this cells acts on its neighboring cells to downregulate the expression of the ligand, thus making adjacent cells following different cell fates. The transcriptional feedback that allows for Notch to regulate its own expression of both receptor and ligand, described previously, is the key mechanism behind pattern formation by lateral inhibition. Lateral inhibition thus makes up an important feature of Notch signaling in neural development that leads to maintenance of the progenitor pool and specification of cell fate in a population of initially homogenous cells. Furthermore, Notch signaling has been implicated in the differentiation of glial cells, neurite development and in homeostasis of the adult nervous system.[65]

The Eph/ephrin signaling pathway is highly involved in the development of the CNS as well. As already mentioned, bi-directional signaling of this pathway is for example involved in processes such as axon guidance and formation of synapses. Moreover, it has been shown that Ephrin-A2 and EphA7 control cell number in the brain by reverse signaling that negatively regulates neural progenitor proliferation.[30] Signaling through Ephrin-A5 and EphA7 has also been shown to be involved in controlling brain size by triggering pro-apoptotic pathways in early cortical progenitors.[66,67]

NEURAL STEM CELLS *IN VITRO*

Expansion of stem cell-like progenitors from the developing CNS *in vitro* offers opportunities to study molecular and cellular processes during development and disease. The model system used in these studies is an adherent monolayer of NSCs derived from the telencephalon from mid-gestation rats. These cells are expanded in serum-free well-defined media in the addition of the mitogen FGF2, and are immunoreactive for the intermediate filament marker nestin. The NSCs have the capacity to differentiate into

neurons, astrocytes, oligodendrocytes and smooth muscle cells through stimulation with Wnt/BMP4, ciliary neurotrophic factor (CNTF), thyroid hormone (T3) or fetal bovine serum/BMP, respectively.[68,69] Withdrawal of FGF2 results in spontaneous differentiation into astrocytes, neurons and to a lower extent, oligodendrocytes. In the presence of FGF2, cells self-renew and maintain their multipotent neural stem cell state. Additionally, FGF2 mediates Notch expression, which in turn inhibits neurogenesis.[70]

Multiple strategies to derive NSCs from pluripotent stem cells have been established. One such method relies on differentiation of NSCs from mouse embryonic stem cells (ESC) in the presence of FGF2 and epidermal growth factor (EGF). Cell lines established from these embryonic stem cell-derived neural stem cells (ESC-NSC) divide symmetrically and express the radial glia marker BLBP and can give rise to neurons, astrocytes and oligodendrocytes in culture.[71]

The 2012 Nobel Prize-winning discovery that adult cells can be reprogrammed to an embryonic stem cell-like state, so-called induced pluripotent stem (iPS) cells, has further opened up for opportunities to manipulate and recapitulate development *in vitro*. As such, NSC cultures have successfully been derived from iPS cells and importantly, this system hold great promise for patient-derived cell cultures to study neurodegenerative disease.

ENGINEERING THE BIOPHYSICAL MICROENVIRONMENT

Bioengineers are making use of a large variety of new materials and techniques in the quest to understand cell function in development and disease. With the gained interest in the field of biophysical cell regulation and mechanotransduction, there is now a vast amount of literature on how to develop cell culture substrates of different elasticities, how to steer cell fate on such materials and how to acquire certain phenotypes that are challenging to derive on rigid plastic dishes. Oftentimes these substrate gels consist of varying degrees of crosslinking of a polymer, and one persisting challenge in the field is how to change stiffness independently of ECM protein concentration. Additionally, tissue elasticity is rarely static but changes with time and disease progression. To recapitulate these events, there are protocols to change substrate stiffness during cell culture.[72,73]

Though not applied in this thesis work, another exciting method of study in the field of cellular bioengineering is 3D cell cultures. Studying cells in two dimensions does not reproduce *in vivo* tissue organization and physiology. Cells naturally exist in three dimensions, and *in vitro* 3D cultures have gained rapidly growing attention. Studies in such systems more closely resemble the *in vivo* scenario, and have generated quite different results than observed in traditional two dimensional *in vitro* studies.[52,74]

Another important cornerstone in the study of cell dynamics is the advent and development of high-resolution techniques that allow for investigation of forces on small scale. Such techniques include optical tweezers, force spectroscopy and atomic force microscopy (AFM), that can be used to measure binding and unbinding events of proteins, molecular interactions at the nanoscale in real time and cell membrane dynamics in living cells.

AIMS

This work addresses the roles of biophysical variables on signaling through cell-ECM and cell-cell communication pathways by utilizing a broad tool kit that includes the development of novel technologies. The **overall goals** of the research presented here were to gain a basic understanding of the roles of biophysical variables on the mechanisms of cell-ECM or cell-cell interactions. Specifically, the respective studies aimed to:

- Use a novel cell culture substrate based on conjugated polymers to study the effect of dynamic FGF presentation to neural stem cells for maintenance of stem cell proliferation and controlled onset of differentiation.

- Elucidate the role of substrate stiffness on Notch signaling and cell dynamics in highly invasive breast cancer cells and control cell dynamics based on Notch activity.

- Develop well-defined ephrin-A5 nano-patterns using DNA origami to investigate the role of ligand spatial distribution on Eph receptor activation in invasive breast cancer cells.

PAPER I

Bioelectronics is an interdisciplinary field of research that combines material science with biology and electronics. One cornerstone in this research area is conjugated polymers. These organic polymers can be in states ranging from semiconductive to highly conductive and have commercial uses such as antistatic coatings, solar panels and light-emitting diodes. The interface between electronic materials and biology has gained huge interest in the field due to the versatility and tunability of these materials. The great advantages of the polymers are their ease of processability, mechanical properties and importantly, the ability to fine-tune the electrical properties. Conjugated polymers have been used to control cell adhesion or neurite outgrowth as well as to deliver ions and biomolecules to cells through organic electronic ion pumps.[75,76,77] We hypothesize that conjugated polymers have the potential to be used as a tool in mimicking the *in vivo* cellular microenvironment.

Organic conjugated polymers contain alternating single and double bonds between the carbon atoms in the polymer backbone, giving rise to a chemical bonding with unpaired electrons. This characteristic enables charge mobility: by insertion of positive or negative electrical charge, which must be balanced by counterions, the polymers can be "doped" to become conductive. Importantly, this structure provides a mechanism to engineer the polymers to suit specific needs. The properties of conjugated polymers are tailored by the synthesis method and the redox state of the polymers. Reversible switching between oxidation states of the polymer can alter bulk properties such as hydrophobicity, conductivity and volume. For instance, when a negatively charged ion is incorporated in the polymer during electrosynthesis and this ion is too large to be mobile, a cation will enter the polymer to maintain charge neutrality during reduction/undoping, which results in a volume expansion. On the contrary, if the ion is small and immobile, it will be released from the polymer upon reduction, resulting in polymer contraction.

In paper I, we explored the biocompatibility of a commonly used conjugated polymer, polypyrrole (PPy), the structure of which is shown in Figure 3. The counterions incorporated upon doping of conjugated polymers have critical significance for their physical and chemical properties as well as their biocompatibility. Thus, the motivation behind this investigation was to identify a PPy-based material that could be used in further studies in which we aimed to recapitulate and control the neural stem cell microenvironment. PPy has been studied with a wide array of counterions for use in neural probes and various kinds of microactuators.[78,79]

In this study, we used PPy electropolymerized with four commonly used counterions to dope the polymer: dodecylbenzenesulfonate (DBS), tosylate (TsO), perchlorate (ClO_4) and chloride (Cl). Although these have been used before in neural probes and interfaces, they had not been previously tested for biocompatibility with primary NSC cultures, which are highly sensitive cell systems. We found that cell survival was correlated with the size of the counterion used: NSCs cultured on PPy(DBS) survived and proliferated in the presence of FGF2, whereas cell survival on PPy(TsO), PPy(ClO4) and PPy(Cl) was minimal.

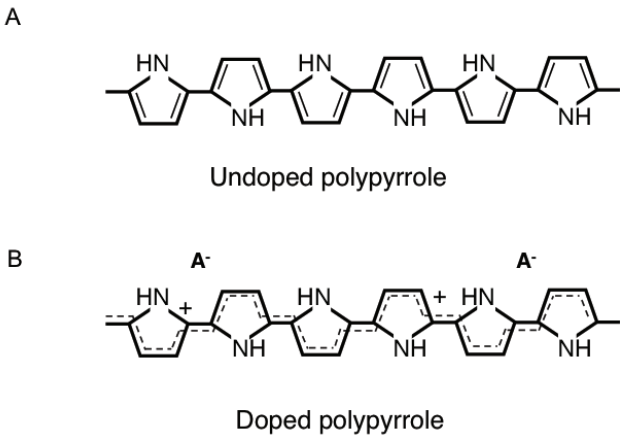

Figure 3. Chemical structures of polypyrrole in the neutral (A) and the doped state (B).

For PPy-based materials to have a relevance in the development of devices or microactuators for PPy/NSC interfaces, these cells must not only survive on the doped, or pristine, polymer but also upon electrochemical reduction. We found that reduction of PPy(DBS) caused a large decrease in cell survival in the cell layer growing on top of the polymer. This was prevented when precoating the PPy film with a gel layer of basement membrane matrix. We hypothesize that this gel creates a buffering layer that inhibits cell death induced by direct or indirect effects of the electrochemical activation of the polymer.

In conclusion, we identified PPy doped with DBS to make a suitable and biocompatible platform for neural stem cell studies. This material is of great interest for future development of PPy-based devices due to its reported stability and large volume changes capabilities of up to 30-40% of the bulk polymer during redox reactions.[80]

PAPER II

Like paper I, the study in paper II is based on organic bioelectronics. A commonly used conjugated polymer in the field is poly(3,4-ethylenedioxythiophene) (PEDOT). Similarly to PPy, PEDOT incorporates negatively charged ions during electrosynthesis to make up for positive charges that arise along the polymer backbone, as illustrated in Figure 4. In addition, it is highly stable and can retain its properties in a broad pH range. As PEDOT itself is insoluble, the common use of poly(styrenesulfonate) (PSS) as a counterion has great advantages, since it not only renders the polymer soluble, but also enhances its conductivity and increases processability. PEDOT:PSS-based materials have been extensively explored for use in sensors and transistors, and are highly compatible with cell culture.[81]

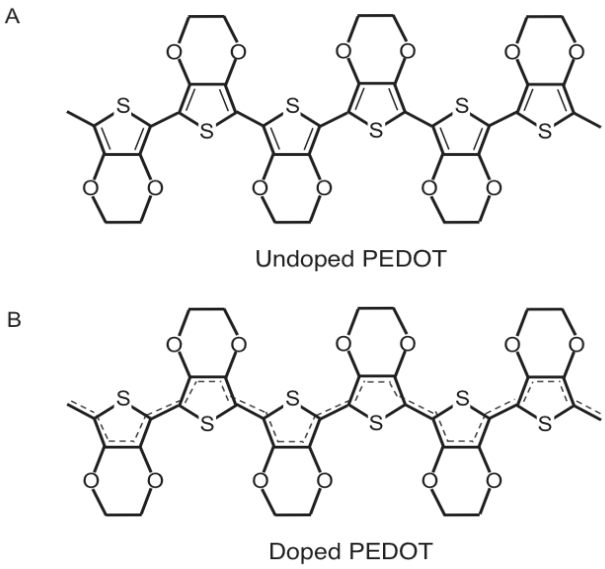

Figure 4. Chemical structures of PEDOT in the neutral (A) and the doped state (B).

In this study we aimed to develop a novel substrate for stem cell culture based on PEDOT, with the unique feature of controlling growth factor presentation. Stem cell culture commonly relies on the sequential addition of mitogens for stem cell proliferation. However, as previously described, growth factors *in vivo* are rarely presented to cells in soluble form, but are instead immobilized on other cells or in the ECM. The rationale behind this study was to more closely recapitulate this aspect of growth factor presentation. Not only does anchored growth factor presentation mimic *in vivo* conditions, but immobilized growth factors have also shown increased protein stability and activity compared to growth factors in solution. Moreover, during embryonic development, growth factor presentation changes over time, a feature that is hard to control in existing growth factor immobilization strategies, which often rely on covalent immobilization.

Several growth factors, including FGF, naturally bind heparin and heparan sulfates. Here we have used the conjugated polymer PEDOT as a means to anchor FGF2, and then used these polymer films as cell culture substrates for expansion of

NSCs. Since heparin and heparan sulfates are negatively charged molecules, we hypothesized that these could be used as counterions in the electrosynthesis of PEDOT. Furthermore, we hypothesized that electrochemical reduction of the conjugated polymer would cause a switch in growth factor presentation, which would decrease or eliminate FGF2 presentation to the NSCs grown on the polymer substrates. As expected, we could demonstrate that FGF2 anchored to PEDOT through heparin supported proliferation of NSCs. Importantly, stem cell properties were maintained over four days of culture without further FGF2 addition, confirming stabilization of the growth factor. In addition, we showed that an electrochemical switch of the polymer substrate triggered spontaneous differentiation of the NSCs into astrocytes and neurons, suggesting that the presentation of FGF2 was reversed by a simple redox reaction.

The tool we developed here more closely mimics the *in vivo* microenvironment during CNS development, where mitogens are presented to cells in an anchored form. This allows for stem cell expansion that can be inhibited at any time to induce the onset of differentiation. The temporal control of growth factor presentation makes this a unique and powerful tool, as does the ability for use with other heparin binding growth factors that altogether could broaden our overall knowledge of stem cell science.

PAPER III

Bridging biology and material science to gain a deeper understanding in cellular and molecular function is at the forefront of biomedicine. This study sought to understand how biophysical attributes in cancerous tissues influence cell state. Matrix stiffness regulates cell behavior, which has been demonstrated for a variety of different cell types, including stem cells and cancer cells. Though the cell culture dish is a routine tool for many cell-based assays, certain physical cues, such as mechanical stimuli exerted on cells in their native habitat, are completely lost in standard culture dishes. For this reason, it is desirable to use biologically relevant elastic substrates for *in vitro* cell culture in order to investigate how cells respond to their physical environment.

While it has been understood that Notch signaling depends on ligand endocytosis in the signaling cell, the exact mechanisms behind these events have remained elusive. After years of speculation, it was recently shown that Notch signaling activation requires an external pulling force applied to the receptor, which exposes the S2 cleavage site for metalloproteases. S2 cleavage is a prerequisite for the next cleavage step by gamma-secretase, which liberates the active NICD. Despite the relative simplicity of the pathway, in which the released NICD is physically involved in gene expression activation by binding DNA through CSL, transcriptional regulation by Notch is not. Rather, it is highly regulated both spatially and temporally. Aberration of the Notch signaling pathway has been strongly linked with malignancy. For instance, Notch signaling is overactive and required for maintenance of the transformed phenotype in human breast cancer. Increased levels of Notch1 and the ligand Jagged1 have been observed in human breast cancer, which in turn correlated with poor prognosis.[60,82]

The requirement for a physical pulling force on the Notch receptor through ligand endocytosis led to speculations that Notch may be mechanosensitive. For instance, Notch has been shown to be under the control of integrin β1 during somite border formation during embryonic development. As integrins are part of cell-ECM adhesion points that transmit forces, these results led to researchers to propose that Notch could be crucial for downstream mechanosignaling. The Notch signaling pathway is also required for the development of blood vessels. It has been shown that blood flow is required for Notch activation suggesting that shear forces exerted in the vasculature directly affect Notch signaling. Furthermore, work has shown that Notch regulates nitric oxide, a signaling molecule involved in mechanotransduction, in the regulation of arterial identity.[83,84] In light of these accumulating results, we were interested in studying Notch signaling in the context of mechanical stiffness sensing. Targeting the Notch signaling pathway is of great interest in the search for new therapeutic strategies in treating breast cancer. Hence, it is crucial to elucidate the biophysical effects and how they influence this signaling pathway.

The stiffness of human tissue is highly diverse, ranging from an elastic modulus of a few hundred pascal to an order of GPa, as measured for pliable, jello-like brain and rigid bone, respectively.[45,85] However, the onset and progression of disease does not only result in biological or functional alterations of tissue, but also structural ones. Tumors are much stiffer than normal tissue, due to increased collagen deposition, ECM remodeling and abnormal matrix turnover. In breast cancer, the tumor stroma has been found to display up to ten times higher stiffness compared to healthy tissue, ranging

from 0.1-0.2 kPa in normal mammary glands to 2 kPa in tumorous mammary tissue. Stiffened, fibrous tissue, as detected on a mammogram, is a hallmark of breast cancer and has been directly coupled with tumor cell invasion and progression.[52]

Mechanical changes have also been observed in individual cells during disease progression, such as in malaria and cancer.[86] Thus, it is of great interest to study cell mechanics in order to increase our understanding of how structural and mechanical characteristics of diseased cells differ from healthy cells. In fact, the investigation of cell mechanics is not new. Already in the late 1940s, Francis Crick used magnetic particles to study the cytoplasm of chick fibroblasts in culture.[87] Nowadays, there are many powerful techniques in place to measure single-cell mechanics. With recent advances, it is now possible to use methods such as microfluidics, optical or laser tweezers or AFM to probe mechanical effects on biological structures, from cells to small molecules. AFM has also been used to test patient-derived cancer cells and tissue *ex vivo*, in an attempt to establish a mechanical profile of cancer.[88,89] Learning about cell mechanics in diseases like cancer may play pivotal role in the development of therapeutics. Additionally, cancer cell mechanical profiles have the potential to be used as biomarkers to detect cancer at early stages.

While AFM has been extensively used to scan surfaces in material science, it has found increasing potential in the field of biology and bioengineering. Force spectroscopy using AFM provides single-cell resolution in cell elasticity measurements. This is done by probing a living cell with a cantilever sensor that measures the mechanical response of the cell upon an applied external force. Specifically, a laser beam is focused on top of a cantilever with a small tip at one end. The reflection of the laser beam is focused onto a photodiode detector, which detects any bending of the cantilever. The cantilever is allowed to move towards an underlying, adherent cell and the tip slightly indents the cell and then retracts. Meanwhile, the force and vertical deflection of the cantilever is measured, resulting in a force-distance curve. The force-curve is then used to calculate the cell's elastic modulus, or Young's modulus. In this study we have used AFM for single-cell force spectroscopy on the highly invasive breast cancer cells MDA-MB-231. Previous elasticity measurements of cancer cells show varying results in stiffness compared to normal cells, which indicates an increased need for standardization of cell mechanics measurements and further investigations.

In line with the overall aim of this thesis work, we have mimicked the mechanical properties of the tumor cell microenvironment here. The objective of this study was to investigate how biophysical cues in the microenvironment of cancerous tissues influence cell state. Also, since it is speculated that Notch is involved in mechanosensing, we wanted to learn whether aberrant Notch activity in breast cancer correlates with tumor stiffness. Therefore, we used synthetic hydrogels made of polyacrylamide as elastic cell culture substrates. The substrates had varying elastic moduli of 0.5 kPa, 2 kPa, 4 kPa, 12 kPa and 50 kPa. In addition to gene expression analysis, we developed a novel Notch signaling detection method based on an *in situ* proximity ligation assay (PLA) to analyze Notch activation in cells. PLA is a powerful technology that extends the capabilities of traditional immunocytochemistry to detect single protein events, such as protein-protein interactions or post-translational modifications of proteins in cells or in tissue. Two primary antibodies raised in different species are used to detect the antigens of interest; in this case anti-NICD and

anti-CSL were used. Specific PLA probes, which are species-specific secondary antibodies attached to short oligonucleotide strands, bind the primary antibodies. If the PLA probes are in close proximity (<40 nm), additional DNA connector probes will hybridize and be ligated to form complete DNA circles. These are then amplified through rolling circle amplification using a polymerase, followed by hybridization of fluorescent probes. The result can be visualized under the microscope as fluorescent, round spots, which are easily quantified. The use of the invasive breast cancer cell line MDA-MB-231 thus allowed us to link Notch signaling to biophysical properties in a malignant breast cancer model. Furthermore, we used AFM to measure cancer cell stiffness in relation to substrate stiffness and Notch signaling.

We found that Notch activity was associated with the elasticity of the underlying substrate, in that Notch levels increased with increasing substrate stiffness. Cell stiffness also increased with increasing substrate stiffness, as well as cell migration. Interestingly, the stiffness of cells could be tuned by regulating Notch signaling, as shown for cells cultured on the softest (0.5 kPa) and stiffest (50 kPa) substrates. Here, cells grown on the soft substrates became stiffer upon Notch activation and cells grown on the stiff substrates became softer upon Notch inhibition. Moreover, we found that Notch inhibition resulted in decreased cell migration on stiff substrates. These results show that targeting Notch signaling levels can directly reduce cell stiffness and cell migration.

Since a cell looses its innate biomechanical properties as soon as it is uncoupled from its native microenvironment, the stiffness of cells measured in culture remains conflicting and does not represent *in vivo* elasticity. In some cases, cancer cells have exhibited a reduction in stiffness compared to normal cells from the same tissue. On the other hand, other studies have reported that elastic moduli increase directly with level of malignancy.[90] One explanation could be that cancerous cells may go undergo stiffness changes during different stages of cancer, much like how cells undergo stiffness changes throughout development. Cells require a highly dynamic actomyosin network for migration along the ECM, which contributes to increased tension and stiffer cells. However, to invade foreign tissue, one can imagine that a soft and flexible cell would be of advantage, in order to squeeze through cell layers and escape.

Clinical trials involving the inhibition of Notch signaling are already ongoing in patients with advanced breast cancer, but this remains to be further exploited. In this study, we aimed to investigate opportunities for cancer therapies that target the cancer cell/microenvironment interface instead of the Notch pathway itself. The lower levels of Notch observed on soft substrates, together with the fact that a reduction in Notch signaling on stiff substrates decreased migration and elasticity, suggest that it may be possible to decrease invasiveness of breast cancer cells by directly targeting the tumor microenvironment.

PAPER IV

In this study, we made use of a novel technique known as DNA nanotechnology. As the name suggests, this method is based on deoxyribonucleic acid, more commonly referred to as DNA, which is the basic building block of life and comprises the genetic code in all living organisms. DNA exists as a double helix, which is formed by the complementary binding between the nucleotide bases thymine and adenine, and cytosine and guanine, more simply known as T, A, C and G, respectively. In DNA nanotechnology, the naturally occurring base-pair interactions are used as a basic principle to self-assemble DNA nanostructures. The idea to use DNA as building blocks to construct nano-size objects was pioneered by Nadrian Seeman in 1982, thus founding the field of DNA nanotechnology.[91] This technique took a huge step forward in 2006 when Paul Rothemund revolutionized this method by introducing a scaffold-based assembly of DNA nanostructures. This kilobase-long ssDNA, usually from a harmless bacteriophage, is folded into any desired shape or pattern by forming complementary base pairing with several hundred short, synthetic oligonucleotide strands, referred to as staple strands. Notably, the genetic information of the DNA sequences are unimportant, as the nucleotide sequences merely are used as building blocks. The work by Rothemund presented a variety of two-dimensional sheets, from stars and smiley faces to world maps, consisting of a single layer of DNA helices. This technique, called DNA origami, was quickly implemented to make complex 3D objects, including stacks, spheres and even nanoboxes with lids.[92,93] Hence, DNA origami has enabled highly controlled design and robust and efficient production of nanometer sized DNA objects.

The desire to use DNA origami for biological research has sparked a great interest in finding applications for this tool. For instance, DNA origami has been used as a nano-ruler for microscopy and to measure distances between single molecules.[94] There is also huge potential for DNA origami to be used as drug delivery vehicles, by specifically binding cells and unloading a cargo of for example anti-cancer drugs.[95,96,97] Other work uses DNA origami in the study of cell and molecular biology, for example to investigate cytoplasmic motor proteins or ion channels.[98,99]

Our motivation to use DNA origami lies in the unique ability to position proteins at precise locations at the nanoscale on the structures. Since the exact position of every staple strand is known, these can be functionalized with other biomolecules, allowing for nanoscale control over protein positioning on the DNA structure. At this point, there exists no other known method that lets scientists tailor-make protein-presenting objects in such a highly controlled manner, at nanoscale distances and independent of protein concentration. We have designed an array of such protein-presenting nanomolecules, of varying proximity and local stoichiometry, which have then been presented to cells. The tool we have developed is a proof-of-principle that cell signaling can be activated using these protein DNA origami structures. More importantly, however, is the identification of functional differences in cells upon cell stimulation using this tool.

Specifically, we were interested in the Eph/ephrin signaling pathway. As previously described, Eph activation depends on the formation of higher order clusters of the ephrin ligand. Furthermore, it has been hypothesized that the degree and effect of Eph receptor activation is determined by ligand distribution on juxtaposed cells. Unfortunately, studying microenvironmental factors, such as ligand-receptor distribution, remains difficult due to lack of control and resolution at the nanoscale. Thus, neither the direct regulation of receptor activation by nanoscale distribution of ligands nor the exact mechanism by which ephrin distribution is translated into biological information is understood. The DNA origami nanostructures combined with protein patterns of well-defined ephrin-A5 that we developed in this study formed a unique tool to probe the roles of spatial distribution of ligands on receptor activation and downstream signaling.

Due to limitations in studying the nanoscale protein arrangements in cells, the sizes of ligand-receptor clusters remain unknown, but it has been suggested that transient macrodomains in the cell membrane, ranging between 10 and 200 nm in size, harbor signaling proteins. The DNA origami-based tool developed is a 140 nm long rod-like structure comprised of 18 double helices. We positioned recombinant ephrin-A5-Fc ligands on this rod, spaced 100 nm or 40 nm apart from one another, as seen in the transmission electron micrographs in Figure 5. Due to the precise ability for patterning of these ligands at the nanoscale, we named this tool *ephrin-A5 nano-calipers* (NC).

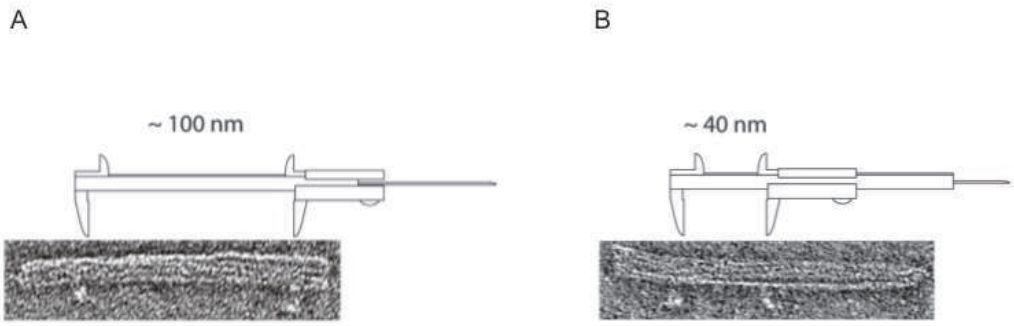

Figure 5. Transmission electron microscopy images showing ephrin-A5 nano-calipers with ligand separation of ~100 nm (A) and ~40 nm (B).

In order to study the effect of ephrin-A5 ligand distribution on Eph receptor signaling, we made use of a highly invasive breast cancer cell line, MDA-MB-231, which overexpresses receptor EphA2. EphA2 is up-regulated in many aggressive cancers, including breast cancer, where it promotes cell migration, and expression levels have been shown to correlate with tumor aggressiveness.[100] Protein tyrosine phosphorylation levels regulate adhesion between cells as well as between cells and the ECM, and elevated levels of tyrosine kinase weakens cell-cell contact and increases adhesion to the ECM. Moreover, overexpression of EphA2 in non-transformed mammary epithelial cells, MCF-10a, induces malignancy and tumorigenic potential.[101] Of note, EphA2 has shown enzymatic activity independent

of ligand binding.[102]

Commonly used methods to study receptor activation include immunocytochemistry or immunoprecipitation followed by immunoblottting. Nevertheless, these methods do not have enough resolution to allow for discrimination between different levels of receptor activation. Therefore, we made use of an *in situ* proximity ligation assay (PLA). Using this method, we were able to specifically analyze EphA2 receptor activation through tyrosine phosphorylation events, which is the first step of receptor tyrosine kinase activation. Specifically, in this assay, the close proximity of EphA2 and phosphotyrosine was visualized as fluorescent dots, which allowed for quantification of Eph receptor activation levels.

In addition to the sensitivity of the PLA, the strength of this assay in our study was the ability to quantify receptor phosphorylation on a single-cell level. Particularly, by culturing the MDA-MB-231 cells on fibronectin micropatterns, we could prevent endogenous activation of the Eph signaling pathway through cell-cell contact. Using this method, we found that receptor activation was indeed triggered by the ephrin-A5 nano-calipers after 15 minutes of stimulation. Moreover, receptor activation by ephrin-A5 spaced 40 nm apart (NC40) was higher than for ephrin-A5 spaced 100 nm apart (NC100). To investigate downstream signaling effects, we performed RNA sequencing, which showed that ephrin-A5 nano-calipers regulated the transcriptome and moreover, that there was a significant differential gene expression upon cell stimulation with NC100 and NC40.

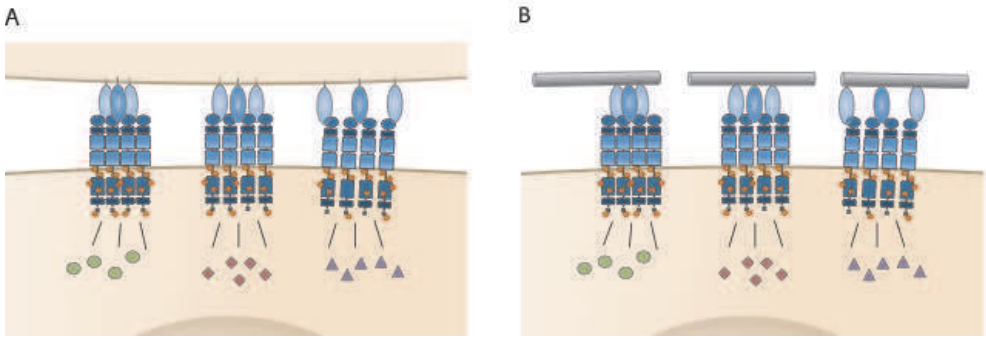

Figure 6. The Ephrin-A5 Nano-Caliper Concept. Ligand-receptor clustering between adjacent cells (A) or regulated by the ephrin DNA origami tool (B). Differences in clustering have been suggested to lead to disparate cell outcomes. The tool developed in this study has the potential to investigate differences in nanoscale ligand-receptor interactions and downstream signaling events.

Further exploring the functional responses of cells treated with our ephrin-A5 nano-calipers involved analysis of invasiveness by commonly used migration assays. Overnight stimulation of the MBA-MD-231 cells with the different ephrin-A5 nano-calipers showed a significant decrease in invasiveness compared to control, and even more so for NC40 than for NC100, which could be correlated to the results from the PLA that showed higher receptor activation for NC40.

The question we asked ourselves in the design of this study was: do receptors on one cell feel and respond to the ligand presentation on other cells? The general idea behind this question is illustrated in 6. Although the nano-caliper tool used here does not represent what really happens in the event of cell-cell contact and ligand-receptor binding, we are confident to say that we have shown that receptors monitor and respond to the spatial distribution of ligands on adjacent cells and that the resulting receptor clusters serve as guidance cues or biological switches that direct the cellular outcome.

CONCLUSIONS

This thesis encompasses studies that make use of a diverse selection of materials, cell systems and signaling pathways. Despite the assorted approaches, all four papers are unified in their application of bioengineering to manipulate cell function in the hopes of gaining a deeper understanding of the roles of biophysical regulation of cell behavior.

FGF, Notch and Eph signaling are the three distinct pathways that this work has focused on. Despite their unique features, it is important to keep in mind that neither one of these pathways is isolated from one another or from other molecular events occurring within and among cells. Entangled crosstalk between signaling pathways is standard and contributes to diversity in cell outcomes. This kind of integration has been observed between FGF, Notch and Eph signaling.[83,103] Importantly, there is a constant interplay of signals, be they physical or chemical, that vary over space and time in a cell-type dependent manner. This is fundamental in embryonic development, where a limited number of signaling pathways are used repeatedly to create a precise pattern of transcription in order to generate various cellular outcomes. As such, reutilization of Notch at two different developmental stages in the same cell can have two distinctly different effects. Addressing the issue of timing of signaling is of great importance when trying to recapitulate the *in vivo* microenvironment, since lineage restriction and other developmental steps in the embryo happen during very limited time windows. Controlling signal timing was the motivation behind **Paper I** and **II**, where we developed an entirely novel neural stem cell culture substrate, which more closely mimics growth factor signaling in the brain during development compared to routine laboratory methods.

Whether the body is in homeostasis or in a diseased state, mechanical stresses constantly bombard the system. From tissue dynamics by contraction and relaxation, to cellular cytoskeleton movement, down to single proteins moving in the lipid membrane, the cells in the body are in constant motion. Cells respond to biophysical forces in the same manner that they alter their reaction to biochemical stimuli depending on when, where and in which cell the signaling occurs. This means that incoming cues from the microenvironment can be translated into different cellular responses. Notably, the stiffness of tissues changes over time during embryonic development and in tumor progression. In light of this, the dynamics of elastic moduli must be investigated in order to understand and control cell function in this kind of environment. This was the goal of **Paper III**, where we aimed to link the stiffness of breast cancer tumors to Notch signaling and cell dynamics. Inhibiting Notch signaling in breast cancer treatment has already been tested in clinical trials. However, Notch inhibition in breast cancer patients remains problematic, partly due to the side effects that come when targeting a common pathway active in other cell types than just cancer cells, and partly because Notch may have tumor suppressive functions. Due to these difficulties, we sought to identify opportunities for cancer therapies that target the cancer cell/microenvironment interface instead of the Notch pathway itself. Our studies demonstrate that it is possible to target cancer cell or matrix dynamics, rather than Notch signaling directly, showing that this kind of therapy could be of great interest in the treatment of breast cancer.

In addition to Notch, the Eph/ephrin signaling pathway offers another promising target in cancer therapy. One way this could be achieved is by blocking forward signaling using either kinase inhibitors or with antibodies or peptides as agonists or antagonists. Research shows, however, that the same Eph receptor can give rise to disparate outcomes[104]. It could thus be preferable to steer cell function by tuning Eph/ephrin signaling, rather than blocking the pathway all together. It has been suggested that receptors in cell membranes are poised to respond to the initial nanoscale spatial arrangement of ligands on adjacent cells and that small differences in this spatial arrangement could lead to disparate cellular responses.

With these hypotheses in mind, we developed a tool using DNA nanotechnology. This allowed us to study ephrin ligand and Eph receptor interactions in order to determine the effect of ligand spatial distribution on receptor activation and downstream events on a nanoscale. The method presented in **Paper IV** is to our knowledge the first that allows for ligand patterning with such precise control. An enormous strength of this novel tool is its ability to be used in 3D culture, since the molecules are used in soluble form. A vast amount of literature has been generated over the past decade that aims to understand physical regulation of cellular state and fate. With the advent of 3D cultures, and techniques being developed to study these, there are many knowledge gaps that can be filled in the near future. Elucidating the mechanisms involved in biophysical regulation of cell function is necessary to understand cellular dysfunction and diseases. 3D cell cultures are likely to give results that more closely capture what happens in tissues *in vivo*. Therefore, we believe that the DNA origami-based tool developed here has great potential in future studies of signaling pathways. In addition, understanding the mechanisms underlying signaling diversity has the potential to be used in the development of cancer therapeutics.

With the accumulating evidence that cells sense and respond to physiological input in the microenvironment, scientists from different fields have come together to answer basic biological questions. A myriad of materials, techniques and engineering approaches are being exploited that aim at controlling cell function by mimicking various aspects of the natural biophysical microenvironment. Recapitulating the *in vivo* stem cell niche has great potential to further what is known about development and tissue regeneration. Likewise, studying the tumor microenvironment in the most realistic setting possible can shed light on biophysical mechanisms involved in tumorigenicity. This research can lead to enormous advancements in the development of cancer therapies.

ACKNOWLEDGEMENTS

I am spoiled to have been surrounded by so many amazing and inspiring people.
Most importantly, Ana, I'm deeply and eternally grateful that you took me on.
Thank you for all the chances I was given and for letting me pursue my passion.
It's been pretty awesome!

Anna, for sharing, caring and your contagious enthusiasm for science. Tack.
Thanks a lot to Dawei, Johan and to my BCF Richie - you are a legend, mate!
To my brilliant super students Ann-Sophie and Ekaterina, my deepest thanks.

Thanks to collaborators and co-authors,
Björn Högberg, Alan, Ferenc and Erik;
Paolo Macchiarini, Philipp, Ylva and Johannes.
It has been a true pleasure working with you all.

Many thanks to the extended family and beyond:
Past and present members of the Hermanson lab - where it all began,
Ola, Aileen, Amilcar, Anna, Erik, Esra, Giulia, Julian, Shirin. Tobias;
Anna Falk, Kelly, Masti, Mari, Mia Lindskog, Jorrit, Kalle and Keira.
Thanks to Agneta Richter-Dahlfors for having been my co-supervisor.

CMB/LICR people who have been along for the ride, in one way or another.
Anders, Heather, Shaobo, Tanya, Urban Lendahl, Evan, Maggie, Sidney, Ze,
Magnus, Vilma, Nigel, Rickard Sandberg and my fellow pub crew members:
Thank you so much for making me love coming to work each and every day.
A special shout-out to everyone in Arne Lindqvist's group. Keep on rocking!

To colleagues turned friends, I am so stoked to have you in my life:
Ingrid, Nina, Frida, Misia, Caroline, Hanna, Cécile, Karin and Noor.
Stockholmsvänner - tack som fan, Krille, Erik, Jennie, B-A och Janne.
Älskade Carolina, Fredrick, Madde, Mari, Johanna, Sara och Charlotta.
Anna, my star, you had me at *My cat's name is Bob, after Bob Dylan*.
Cajsa, tänk att du kom tillbaka! Jag vore ingenstans om du inte fanns.
Helena, min bästis varje dag, vi gå över dagsstänkta berg, du och jag.
Thank you all from the bottom of my heart.

Tack min finaste Jeja och Johan för att ni alltid finns där.
Till mamma och pappa för allt ni gett mig
och för att ni alltid låtit mig drömma fritt,

Danke.

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